CHALLENGES AND RECOMMENDATIONS FOR ADVANCING EXPOSURE SCIENCE

A principal objective of improving exposure science is to build confidence in exposure estimates by addressing or reducing uncertainty in the estimates used to support risk-based decision-making. That objective is best met by developing and further integrating monitoring, measurement, and modeling efforts and by harmonizing exposures among test systems, the multimedia environment, and humans. Incrementally increasing the number of chemicals included in monitoring programs can help in evaluating and refining exposure models and in developing new approaches to integrate exposure data and constitutes an initial and pragmatic path. However, increased environmental monitoring alone will not be sufficient to improve exposure science. Interpreting the monitoring data and appropriately applying exposure data in risk-based evaluations will require continued complementary development and evaluation of exposure-assessment tools and information, such as fate and transport models, PBPK models, and data on chemical quantity and use, partitioning properties, reaction rates, and human behavior.

In this section, challenges and recommendations to advance exposure science are discussed further. The points include some guidance initially presented in the ES21 report and some new, more pragmatic points, specifically related to the application of exposure science to risk-based evaluations. The points build on the advances and applications detailed in this chapter, which present key development opportunities for the field recommended by the committee. Generally, the recommendations and challenges cover a continuum: preparation of infrastructure, collection of data, alignment of exposures between systems, integration of exposure data, and use of data for priority-setting. The ES21 Federal Working Group (EPA 2016b) is particularly well-positioned to coordinate and support the recommendations outlined below by further strengthening federal partnerships for the efficient development of exposure-science research and by engaging with other stakeholders to address the challenges that face the development and application of exposure information for risk-based evaluations. The committee notes that several recommendations below call for developing or expanding databases. In all cases, data curation and quality evaluation should be a routine part of database development and maintenance.

Expand and Coordinate Exposure Science Infrastructure to Support Decision-Making

Challenge: A broad spectrum of disciplines and institutions are participating in advancing exposure methods, measurements, and models. Given the many participants in exposure science, most information is fragmented, incompletely organized, and not readily available or accessible in some cases. Thus, the full potential of the existing and emerging information for exposure-based and risk-based evaluations cannot be realized. The committee emphasizes that the rapid growth in exposure science presents unprecedented opportunities for more efficient, complete, and holistic use of exposure information, especially if the information can be well organized into a readily accessible format.

Recommendation: An infrastructure for exposure information should be developed to organize and coordinate better the existing and rapidly evolving components of exposure science and ultimately to improve exposure assessment. The infrastructure should be organized by using conceptual and systems-based frameworks that are commonly used in exposure assessment and should facilitate the generation, acquisition, organization, access, evaluation, integration, and transparent application and communication of exposure information. The infrastructure might best be comprised of an Internet-based network of databases and tools rather than one database and could expand on existing infrastructure and databases. Guidance for generating, evaluating, and applying exposure information (WHO 2005; EPA 2009) should be expanded to enable inclusion of data in the databases.

Recommendation: Coordination and cooperation should be encouraged among the large network of agencies, institutions, and organizations that produce and use exposure information for different but ultimately connected and complementary objectives. Cooperation should increase the efficiency with which the infrastructure described above is developed, and a common ontology of exposure science (Zartarian et al. 2005; Mattingly et al. 2012; EPA 2016b) should continue to evolve to facilitate interdisciplinary communication in the development and application of exposure information.

Identify Chemicals or Other Stressors and Quantify Sources and Exposures

Challenge: Nontargeted analysis in environmental and human media indicates that there are many unknown chemicals in complex uncharacterized mixtures to which humans are exposed. Analytical methods and standards are not available for most chemicals and degradation products, and this hinders the capacity to identify and quantify chemical exposures. Furthermore, uncertainty in source information—product composition, chemical quantity, use, and release rate—is a major obstacle to exposure estimation for most chemicals.

Recommendation: Current efforts to obtain and organize information on chemical quantities in and rates of release from products and materials, particularly consumer products and materials in the indoor environment, should be expanded substantially. Curated databases that contain analytical features that can be used in chemical identification should be expanded, and increasing the availability of analytical standards for chemicals and their degradation products should have high priority. Ultimately, the capacity to conduct targeted and nontargeted analyses to identify and quantify new and existing chemicals and mixtures in environmental media and humans should be increased.

Improve Knowledge of Processes That Determine Chemical Fate in Systems

Challenge: Understanding the influence of processes that control the fate, transport, and ultimately concentration of chemicals in environmental compartments and in animal and cell-based test systems is essential for characterizing and predicting exposures. Information on system properties, processes, and transformation pathways that contribute to chemical exposure is nonexistent, incomplete, and inconsistent, and this limits the capacity for more comprehensive, quantitative exposure-based and risk-based evaluations.

Recommendation: Databases of chemical properties and information on rates and processes that control chemical fate in vitro, in vivo, and in environmental systems should be developed. Information is needed, for example, on partitioning (distribution) coefficients, degradation and transfer rates, and metabolic and environmental transformation pathways. Information might be obtained through experiments or modeling.

Recommendation: Methods for measuring and predicting chemical transformation pathways and rates in environmental media, biological media, and biological test systems should be developed and applied. The methods should be used to quantify human exposures to chemical mixtures (parent chemicals and metabolites) over time and to interpret results from test systems in the context of actual human exposures. In particular, knowledge of environmental, human, and test-system properties and conditions that influence exposures should be improved. Human pharmacokinetic data on metabolism, chemical transporters, and protein binding should be generated for chemicals in consumer products and food-related applications to improve the interpretation of human biomonitoring data from urine, blood, and emerging matrices.

Align Environmental and Test-System Exposures

Challenge: Aligning environmental exposures and information obtained from experimental systems is a critical aspect of risk-based evaluation and is required for improving environmental epidemiology. Various units of quantification, such as administered or unmeasured dose, are often applied, and assumptions, such as steady-state or equilibrium conditions, are made. However, pharmacokinetic and fate processes and other factors often confound the interpretation and translation of exposure information between humans and the environment and experimental systems.

Recommendation: Concentrations in the test-system components should be quantified over time by measurement, which is preferred, or with reliable estimation methods. Methods and models that explicitly translate quantitative information between actual exposures and test-system exposures should be developed and evaluated.

Recommendation: Chemical concentrations that reflect human exposures as derived from biomonitoring measurements or from predictive exposure models should be considered when designing testing protocols for biological assays. Improving knowledge of processes that determine chemical fate in biological and test systems will be necessary to meet this recommendation.

Integrate Exposure Information

Challenge: Integration and appropriate application of exposure data from environmental media, biomonitoring samples, conventional samples (blood and urine), and emerging matrices (hair, nails, teeth, and meconium) is a scientific, engineering, and big-data challenge. The committee emphasizes that integration of measured and modeled data is a key step in developing coherent exposure narratives, in evaluating data concordance, and ultimately in determining confidence in an exposure assessment.

Recommendation: New interdisciplinary projects should be initiated to integrate exposure data and to gain experience that can be used to guide data collection and integration of conventional and emerging data streams. The projects might start as an extension of existing cooperative projects among federal and state agencies, nongovernment organizations, academe, and industry that focus on integrating measurements and models for improved quantitative exposure assessment. High priority should be placed on extending existing (EPA, CDC, and WHO) guidance on quality of individual exposure data and assessments to include weighing and evaluating the quality of integrated experimental and modeled information from multiple matrices and data streams.

Determine Exposure-Assessment Priorities

Challenge: All the many uses of exposure data—from selection of chemicals for use in new products to risk-based decision-making to exposure ranking—require exposure data, often for thousands of chemicals, over time and space. Whether or not analytical methods are available for the chemicals, the resources and time that are required for direct measures of exposure are not available, and resource-intensive, high-confidence exposure measurements might not be necessary in some cases. A key challenge for exposure science is how best to focus resources on the highest-priority chemicals, chemical classes, mixtures, and exposure scenarios.

Recommendation: Continued development of computational and experimental tools that maximize the value of existing knowledge for estimating exposure should have high priority. Those approaches might initially focus on selected near-field exposures that are known to be important, on chemical classes that are of high interest because of data on biological effects, or on other objectives, such as exposure ranking of members of a chemical class that are being investigated for use in new products.

Recommendation: Continued development of approaches for exposure-based priority-setting that use uncertainty analysis to establish and communicate levels of confidence to support decision-making should be encouraged. The need to improve models or data that are used for priority-setting should be evaluated on the basis of the level of uncertainty and the tolerance for uncertainty in the decision-making context. Uncertainty and sensitivity analyses should guide selection and priority-setting among data gaps to be filled.

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3

Advances in Toxicology

The decade since the publication of *Toxicity Testing in the 21st Century: A Vision and a Strategy* (NRC 2007) has seen continued advances in an array of technological and biological tools used to understand human function and disease at the molecular level. Some advances were initially catalyzed by the Human Genome Project, which of necessity required technological innovations and large-scale collaborations to reach the ultimate goal of mapping the sequence of DNA. Other developments came from advances made by the pharmaceutical industry to screen for chemicals that have specific biological functionality but minimal off-target effects. As a result of those advances, an era of big-data development and of public access and data-sharing has arrived with ever-increasing data-storage capacity, computational speed, and open-access software. Research has also become more multidisciplinary; project teams today often include geneticists, toxicologists, computer scientists, engineers, and statisticians.

A number of advanced tools can now be used in toxicological and epidemiological research; some examples are listed below.

- Large banks of immortalized cells that are derived from lymphocytes and collected from different populations worldwide are available for toxicological research.
- Genetically diverse mouse strains have been created by a multi-institution collaboration (the Complex Trait Consortium; Threadgill and Churchill 2012) and are available for medical and toxicological research. They have been fully genotyped because of the relatively low cost of sequencing today, and the sequence information is publicly available.
- Microarrays and next-generation RNA sequencing can reveal postexposure changes in the simultaneous expression of large numbers of genes (the transcriptome). Technologies are also now available to profile the epigenome (epigenetic changes, such as methylation and histone modifications), the proteome (proteins present in the cell), and metabolome (small molecules).
- Large compilations of a wide variety of biological data are publicly available, as is software for data access, interpretation, and prediction. Text-mining tools applied to scientific-literature databases provide approaches for developing hypotheses on relationships between chemicals, genes, and diseases.
- Automated systems that use multiwell plates provide a high-throughput platform for measuring a wide array of effects in cells and cellular components in response to chemical exposures. Automated, multiwell testing can also be applied for rapid testing of zebrafish, vertebrates that are relatively genetically homologous with humans.
- Computational advances have enabled the development of chemical-structure–based methods for predicting toxicity and systems-biology models for evaluating the effects of perturbing various biological pathways.

Some of the advanced tools could be used to address issues in toxicology and ultimately risk assessment (see Chapter 1, Box 1-3). Some of the general risk-assessment questions to which the tools could be applied are the following:

- Planning and scoping: Which chemicals should undergo comprehensive toxicological evaluation first (that is, how should priorities be set among chemicals for testing)?
- Hazard identification: What adverse effects might a chemical have? For example, could it pose a carcinogenic risk or affect kidney or reproductive function? If a data-sparse chemical has a structure or

biological activity that is similar to that of a well-studied chemical, can the same types of toxicity be assumed and, if so, at similar exposures? Are cellular-assay responses adaptive (or inconsequential) or harbingers of adverse effects in humans? Does the chemical operate through the same pathways or processes that are associated with cancer, reproductive toxicity, or other adverse human effects?

- Dose–response assessment: How does response change with exposure? At what exposures are risks of harm inconsequential? Is there a threshold exposure at the population level below which there is no adverse effect?

- Mixtures: What are the hazards and dose–response characteristics of a complex mixture? How does the addition of a chemical to existing exposure contribute to risk?

- Differential susceptibility and vulnerability: Are some populations more at risk than others after exposure to a specific drug or environmental chemical? For example, are some more susceptible because of co-exposures, pre-existing disease, or genetic susceptibility? Are exposures of the young or elderly of greater concern?

Those risk-assessment questions provide the backdrop for considering the recent advances in toxicological tools. Information obtained with the new tools can advance our understanding of the potential health effects of chemical exposures at various points along the exposure-to-outcome continuum, shown in Figure 3-1 below. The starting point along the continuum is the transformation of external exposure to internal exposure, which was discussed in Chapter 2 of this report (see Figure 2-1). The ultimate goal is prediction of the response of the organism or population to exposure, and different tools can be used to probe or inform different places along that continuum. As noted in Chapter 2, although the continuum is depicted as a linear path, the committee recognizes that multiple interconnecting paths are typically involved in the continuum.
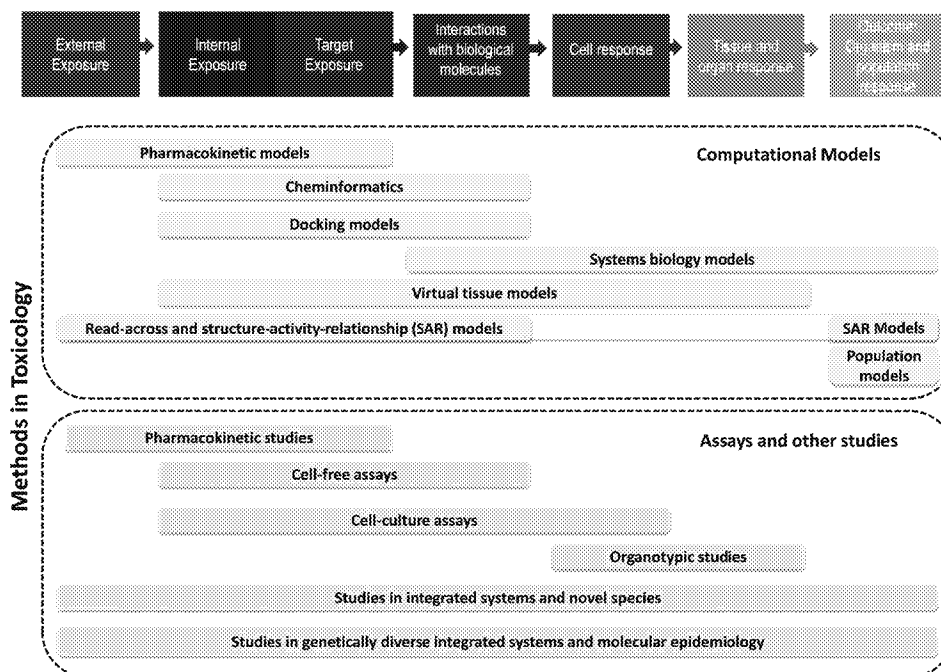

FIGURE 3-1 Computational models and biological assays are shown with the exposure-to-outcome continuum to illustrate where the models and assays might be used to provide information at various points in the pathway. The clear portion of the bar for read-across and SAR models reflects the fact that connections are typically made between analogous chemicals for either the initial biological effect or the outcome. However, biological tools can also probe the response at the cell or tissue level and provide support for read-across and SAR analyses. If sufficient data are available, read-across and SAR analyses can be performed at various points along the exposure-to-outcome continuum.

This chapter describes a variety of new assays and computational tools that are available for addressing risk-based questions, but it is not meant to be comprehensive. The chapter organization follows the progression along the exposure-to-outcome continuum; the discussion begins with assays and computational tools that are relevant for probing interactions of chemicals with cellular components and ends with ones that are relevant for predicting population-level responses. Understanding of pharmacokinetic relationships is critically important in toxicological evaluations for many reasons—for example, to evaluate whether exposures in in vitro cultures and in vivo assays are similar in magnitude and duration to exposures that result internally in exposed humans; to extrapolate from high to low dose, from one exposure route to another, and between species; and to characterize variability in internal human dose associated with a given exposure. Advances in pharmacokinetic analyses and models were discussed in Chapter 2 and are not elaborated on further here. The chapter concludes with a discussion of challenges and offers recommendations that should help to address the challenges.

The committee emphasizes that most Tox21 assays or systems were not developed with risk-assessment applications as an objective. Therefore, understanding on how best to apply them and interpret data in a toxicology context is evolving. For example, assay systems that were designed to detect agents that have high affinity for or potency against a particular biological target might not be optimized to detect agents that have moderate or low potency or that cause more than one effect. Some risk questions are being addressed as data from high-throughput systems become more available. However, the usefulness or applicability of various assays will need to be determined by continued data generation and critical analysis, and some assays that are highly effective for some purposes, such as pharmaceutical development, might not be as useful for risk assessment of commodity chemicals or environmental pollutants.

PREDICTING AND PROBING INTERACTIONS OF CHEMICALS WITH CELLULAR COMPONENTS

Chemical interactions with specific receptors, enzymes, or other discrete proteins and nucleic acids and promiscuous interactions, such as those between an electrophile and a protein or DNA, have long been known to have adverse effects on biological systems (NRC 2000, 2007; Bowes et al. 2012). Accordingly, the development of in vitro assays that probe molecular-level interactions of chemicals with cellular components has been rapid, driven partly by the need to reduce high attrition rates in the drug-development process. Although various new assays have been developed, only a single assay—one that evaluates the human potassium channel (hERG channel)[1]—has been integrated into new drug applications. Figure 3-2 illustrates some typical interactions with cellular components, and the following sections describe how the interactions are being investigated.

Predicting Interaction by Using Chemical Structure

In recent years, predicting chemical interactions with protein targets on the basis of chemical structure has become much more feasible, particularly with the development and availability of open-access data sources (Bento et al. 2014; Papadatos et al. 2015). There are many published examples of computational models that have been developed to predict the interaction of a molecule with a single protein, most notably models for predicting hERG activity (Braga et al. 2014) and interaction with the estrogen receptor (Ng et al. 2015), but prediction of multiple interactions in parallel is now possible given available computational power. For example, Bender et al. (2007) used chemical similarity to predict the protein–chemical interactions associated with a novel chemical structure with a reported average accuracy of over 92% with some proteins and high selectivity; that is, only small numbers of active predictions were later shown to be negative in vitro. Although most of the activities were predicted correctly, it was at the expense of a

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[1]The blockade of hERG channel has been directly implicated in prolongation of the QT interval, which is thought to play a role in the potentially fatal cardiac arrhythmia torsades de pointes.
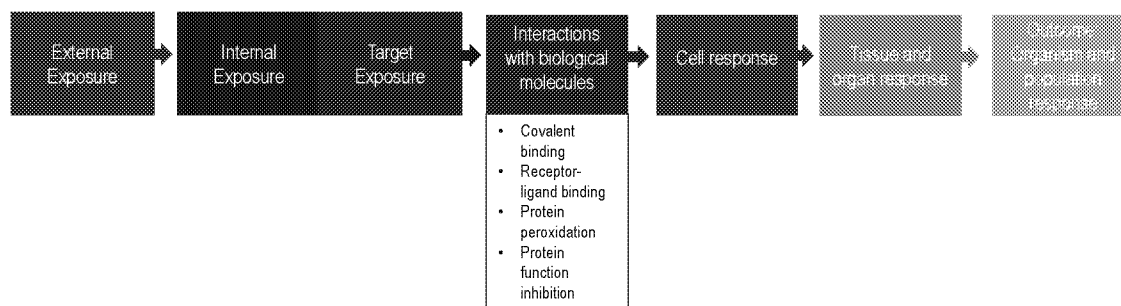
FIGURE 3-2 Exposure-to-outcome continuum with examples of types of interactions between biological molecules and chemicals.

high false-positive rate (that is, large numbers of inactive chemicals were predicted to be active). Most of the models have been built by using pharmaceutical candidates that have a high affinity for the particular protein, but there are examples in the literature in which the same approaches have been applied to identify chemicals that bind a receptor with low affinity (see, for example, Hornung et al. 2014).

Research to improve the prediction of protein–chemical interactions continues apace. Lounkine et al. (2012) used the similarity-ensemble approach—a method first published by Keiser et al. (2007)—and predicted the activity of 656 marketed drugs with 73 protein targets that were thought to be associated with clinical adverse events. The authors reported that about 50% of the predictions of activity were later confirmed experimentally with binding affinities for the protein targets of 1 nM to 30 μM. Cheng et al. (2012) evaluated chemical–protein interaction sets that were extracted from the ChEMBL database[2] by using a computational method, multitarget quantitative structure–activity relationship (QSAR), that evaluates G-protein coupled receptors (GPCRs) and kinase protein targets. Sensitivities were reported to range from 48% to 100% (average, 84.4%), and specificity for the GPCR models (about 99.9%) and the kinases was high.

Assessing Interactions with Cell-Free Assays

Cell-free or biochemical assays have long been used to probe the interactions of chemicals with biological molecules, such as enzymes and hormone receptors, and their activity with these specific targets (Bhogal et al. 2005). The assays can provide reliable and valid results with high agreement between laboratories and can be applied in low-, medium-, or high-throughput formats (Zhang et al. 2012a).

The US Environmental Protection Agency (EPA) is exploring the use of the commercially available cell-free assays, run in high-throughput format, that were originally developed for preclinical drug evaluation to assess environmental chemicals (Sipes et al. 2013). The panel selected by EPA measures various activities, including binding to GPCRs, steroid-hormone and other nuclear receptors, ion channels, and transporters. The panel also covers activation of kinases, phosphatases, proteases, cytochrome P450, and histone deacetylases (Sipes et al. 2013). Roughly 70% of the assays are derived from human cells, 20% from rat cells, and the remainder from other species.

A wide variety of cell-free assays that evaluate other targets have been developed and are being used in pharmaceutical, biomedical, and academic laboratories (Xia et al. 2011; Mehta et al. 2012; Landry et al. 2015; McKinstry-Wu et al. 2015). They are being used to probe a wide array of protein types and functions, such as nod-like receptors, which are involved in immune and inflammatory responses (Harris et al. 2015), methyltransferases (Dong et al. 2015), and various membrane proteins (Wilcox et al. 2015).

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[2]ChEMBL is a chemical database of biologically active molecules that is maintained by the European Bioinformatics Institute of the European Molecular Biology Laboratory.

The potency of the chemical's interaction in vitro—measured, for example, as an IC_{50} or KI[3]—provides information on the likelihood of an in vivo concentration high enough to permit observation of the phenotypic response. The degree of inhibition or activation of the protein function that is required for a phenotypic response to be observed can vary widely and will depend partly on the nature and function of the protein or enzyme. For inhibitors of GPCRs, the anticipated pharmacological response has been observed in vivo at plasma concentrations less than or equal to 3 times the measured IC_{50} of the chemical in question when corrected for plasma-protein binding (McGinnity et al. 2007). As a rule of thumb for pharmaceuticals, a 100-fold margin between the measured IC_{50} or KI in a cell-free assay and the circulating plasma unbound C_{max} has been considered adequate to represent minimal risk of toxicity (N. Greene, AstraZeneca, personal commun., December 14, 2015). However, for environmental chemicals, which are not tested in clinical trials or followed up through medical surveillance, a different rule of thumb might be appropriate. And it is important to remember that toxicity is influenced by many factors, including the required degree of receptor occupancy, the ability of the chemical to reach the site of action (for example, to penetrate the blood–brain barrier), the nature of the modulatory effects (for example, inhibitor, agonist, or allosteric modulator), the kinetics of the binding of the interaction with the receptor, and exposure duration.

CELL RESPONSE

Cell-based in vitro assays have existed for nearly a century; the first publication of a dissociated cell culture was in 1916 (Rous and Jones 1916). Cell-culture technology has evolved to the point where many cell lines are available and more can be produced with current techniques. Cell cultures provide easy measurement of gene and protein expression and a variety of potentially adverse responses (see Figure 3-3) and can be scaled to a high-throughput format (Astashkina et al. 2012). Additionally, cell-based assays derived from genetically different populations can allow rapid assessment of some aspects of variability in response to chemical exposures that depend on genetic differences (Abdo et al. 2015).

Cell-based assays are being used to inform hazard identification and dose–response assessments, mostly as a complement to data from whole-animal or epidemiological studies to address questions of biological plausibility and mechanisms of toxicity. For example, in evaluations of chemical carcinogenicity, the International Agency for Research on Cancer (IARC) gives weight to functional changes at the cellular level (IARC 2006) and considers the relevance of the mechanistic evidence with regard to key characteristics of cancinogens (Smith et al. 2016). Cell-based assays have been critical in the IARC assessments (IARC 2015a,b). Human-derived and animal-derived cell cultures have also been used to discern dose–response relationships and toxicogenomic profiles, for example, for ethylene oxide responses (Godderis et al. 2012). The assays have potential use in addressing many of the risk-based questions raised at the beginning of this chapter and as illustrated in Chapter 5.
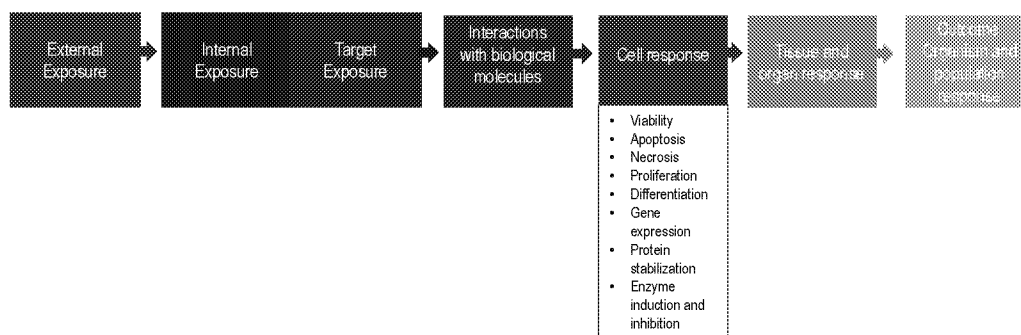

FIGURE 3-3 The exposure-to-outcome continuum with examples of cell responses.

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[3]IC_{50} is the concentration required to cause 50% of the maximal inhibitory effect in the assay, and KI is the inhibition constant for a chemical and represents the equilibrium constant of the dissociation of the inhibitor-bound enzyme complex.

Cell cultures can be grown in a variety of architectures, including monolayer and 3-D cultures of cell lines, and can be used as indicators of possible tissue, organ, and sometimes organism-level signs of possible toxicity, particularly in integrated systems that consider effects and signaling among cell types (Zhang et al. 2012a). They can be used to evaluate a number of cellular processes and responses, including receptor binding, gene activation, cell proliferation, mitochondrial dysfunction, morphological or phenotypic changes, cellular stress, genotoxicity, and cytotoxicity. Various techniques and measurements—such as impedance, gene transcription, direct staining, reporter-gene output, and fluorescence or bioluminescence resonance energy transfer—can be used to measure cellular responses and processes (An and Tolliday. 2010; Song et al. 2011; Asphahani et al. 2012; Smith et al. 2012). Furthermore, simultaneous measurements of multiple toxic phenotypes are possible with high-content imaging and other novel techniques. This section describes some of the recent developments in using cell-based assays to evaluate cellular response and emphasizes advances that can improve toxicology and risk assessment.

The committee notes that cell-based assays have some limitations; one key concern involves metabolic capabilities. Specifically, do the assays capture how exogenous substances are metabolized in the body? That particular limitation might not be a concern for assays that are performed with low-throughput methods in which it might be possible to determine a priori whether metabolism is important for toxicity and, if so, to find ways to test the metabolites in addition to the parent chemicals. However, little or no metabolic capacity is a particular concern for high-throughput systems that are used for priority-setting. Parent chemicals and metabolites can differ substantially in toxicity and potency. If the in vitro assays do not sufficiently capture critical metabolites that form in humans, they might not give valid results for assessment because they are not testing the chemicals that potentially give rise to toxicity. Furthermore, although some assay systems might capture metabolism in the liver, extrahepatic metabolism might be the driver of some chemical toxicity, so the spectrum of relevant in vivo metabolic activation is an important consideration in understanding the validity of in vitro studies and interpreting the results from both in vitro and in vivo studies. EPA, the National Institute of Environmental Health Sciences, and the National Center for Advancing Translational Sciences are awarding research grants to make progress on the issue. For example, a multiagency collaborative announced in 2016 a $1 million competition in the Transform Tox Testing Challenge: Innovating for Metabolism; the challenge called on innovators to identify ways to incorporate metabolism into high-throughput screening assays (EPA/NIH/NCATS/NTP 2016). EPA is also attempting to develop a system that encapsulates microsomal fractions of human liver homogenate in a matrix, such as an alginate, that will allow diffusion of low-molecular-weight chemicals but retain the toxic lipid peroxides. As an alternative approach, EPA is attempting a method that would transfect cells with mRNAs of enzyme-encoding genes to increase metabolic transformation intracellularly. The committee views those initiatives as steps in the right direction and emphasizes the importance of addressing the issue of metabolic capacity.

Primary Cells

Primary cells are isolated directly from fresh animal or human tissue. They can be obtained from a wide variety of tissues, such as liver, brain, skin, and kidney; and they are amenable to high-content screening and analysis (Xu et al. 2008; Zhang et al. 2011; Thon et al. 2012; Raoux et al. 2013; Tse et al. 2013; Valdivia et al. 2014; Feliu et al. 2015). Although primary cells are more reflective of in vivo cellular and tissue-specific characteristics than are immortalized cells (Bhogal et al. 2005), they can be short-lived in culture and suffer from rapid dedifferentiation within hours to days.

Several approaches to adapt primary cell culture to a high-throughput format for chemical-toxicity testing have been made (Sharma et al. 2012; Berg et al. 2015). For example, EPA profiled over 1,000 chemicals (Houck et al. 2009; Kleinstreuer et al. 2014) to identify activity in eight primary cell systems, including ones that used fibroblasts, keratinocytes, and endothelial, peripheral blood mononuclear, bronchial epithelial and coronary artery smooth muscle cells. With proprietary software, chemicals were clustered by bioactivity profiles, and some possible mechanisms of chemical toxicity were identified. The lack of publicly available datasets with which to compare the results and the complexity of the resulting

data precluded sensitivity and specificity calculations (Kleinstreuer et al. 2014). The standard by which to judge construct validity—that is, whether an assay system as a whole adequately represents the target biological effect—still poses a challenge for these and other assays described in this chapter (see "Challenges and Recommendations for Advancing Toxicology" later in this chapter).

A major advance in primary cell culture over the last decade is the development of 3-D cultures of cell lines.[4] 3-D cell cultures have better behavior and function than the monolayer cultures (van Vliet 2011) and are of increasing interest in the development of cancer drugs because they recapitulate the tumor microenvironment to a much greater extent than do conventional monolayer assays that use a flat layer of cells (Edmondson et al. 2014; Lovitt et al. 2014). A number of assays that use 3-D cultures of primary cells from various tumors have been developed. Several studies (Arai et al. 2013; Chen et al. 2014) have shown some degree of drug resistance to well-characterized cancer drugs, depending on assay type; 3-D assays show greater drug resistance.

Similarly, primary isolated hepatocytes are the most widely used for in vitro testing, and 3-D culture systems with added cofactors are being developed to overcome limitations of conventional monolayer systems (Soldatow et al. 2013), which notably include lack of sensitivity for detection of hepatotoxic drugs. The 3-D cultures that are used, for example, for enzyme induction or inhibition studies, maintain function for a relatively long period (1–3 days) and can be used to re-establish cellular polarity that is lost in monolayer cultures. Advances in liver-culture techniques and technology have led to improvements and greater complexity in 3-D liver-cell culture for use in toxicological evaluations, and the next step is development of a bioartificial liver, commonly referred to as an organ-on-a-chip, discussed in greater detail below in "Tissue and Organ-Level Response".

The examples of tumor-cell and liver-cell cultures discussed in this section highlight the movement from monolayer cultures to improved 3-D cultures of greater complexity and ultimately toward organotypic models for various tissues and organs (Huh et al. 2011; Bulysheva et al. 2013; Guiro et al. 2015).

Immortalized Cell Lines

Immortalized cell lines can be derived from isolated human cancer cells or from primary cells that have been genetically altered for enhanced longevity and resilience in tissue culture. Immortalized cell lines do not need to be isolated and harvested for each use, are relatively easy to maintain and propagate, are stable when replated multiple times, and can be easily frozen and shared between laboratories and grown in large quantities. Cloning immortalized cells enables testing in genetically identical cells, and immortalized cell lines that are derived from diverse populations allow inquiry into the variability of chemical toxicity among populations (Abdo et al. 2015). However, more than the conventional monolayer cultures of primary cells, immortalized cell lines can lose native in vivo properties and functionality. They can have altered cellular polarity (Prozialeck et al. 2003; Soldatow et al. 2013), non-native genetic content (Yamasaki et al. 2007), and decreased amounts of key cellular features (such as ligands, transporters, and mucin production); and they can be contaminated with other cell lines, such as HeLa and HepG2. Alterations in cellular phenotype can result in insensitivity to and mischaracterization of test chemicals. For example, when testing the difference between mitochondrial toxicity observed in renal proximal tubule cells (primary cells) and that observed in immortalized human renal cells, researchers found that primary cells were capable of identifying more possible toxicants than were immortalized cell lines (Wills et al. 2015).

Many of the assays in the federal government's ToxCast and Tox21 programs use immortalized carcinoma-derived cell lines (T47D breast, HepG2 liver, and HEK293T kidney). The assays have shown potential for identifying chemical carcinogens found in rodents (Kleinstreuer et al. 2014) and for exhibiting some predictive ability in the preliminary classification of hepatotoxic chemicals in guideline and guideline-like animal studies (Liu et al. 2015). However, the assays have also been shown to be unable to predict some well-recognized hazards observed in humans or animals (Pham et al. 2016; Silva et al. 2015).

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[4] *3-D culture* is a generic term that is used to describe culture systems that are grown on some sort of support or scaffold, such as a hydrogel matrix. 3-D cultures often have two or more cell types.

ToxCast data have been proposed for use in predicting in vivo outcomes of regulatory importance (see Rotroff et al. 2013; Sipes et al. 2013; Browne et al. 2015), such as estrogenic properties of chemicals predicted by the uterotrophic assay, but their use as replacement assays has been the subject of research and discussion. For example, EPA's Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) Science Advisory Panel recommended that the agency not replace the uterotrophic assay with a computational model of estrogen receptor agonist and antagonist activity derived from ToxCast data (EPA 2014a). Although the panel noted a number of strengths of the model, it had concerns about diminished performance of the model for nonreference chemicals and the inability of the model to assess chemicals that had modified toxicity because of pharmacokinetic factors or that had toxicity pathways different from those evaluated in the assays. Thus, the panel found that further research was needed. More recently, EPA reconsidered the results of a high-throughput battery of estrogenicity assays, concluded that the test battery is a satisfactory replacement of the uterotrophic assay for tier 1 endocrine-disrupter screening, and intends to use the results of the test battery to evaluate and screen chemicals in the future (Browne et al. 2015; EPA 2015).

Because immortalized cell lines are limited in the degree to which they can represent cells in intact tissues, alternative approaches of cell immortalization have been developed and are now being made commercially available. "Conditionally immortalized" cell lines that can undergo differentiation are increasingly available for use in biomedical research with potential applications in toxicology (Liu et al. 2015).

Stem Cells

Advances in stem-cell research have allowed the generation of a wide array of cell types, some of which have metabolic competence, which makes them useful for studying the effects of chemicals on various tissues (Scott et al. 2013; Gieseck et al. 2015). Fit-for-purpose stem-cell–based tests are becoming commercially available (Anson et al. 2011; Kolaja 2014), and research is under way to develop stem cells for application in toxicology (Sjogren et al. 2014; Romero et al. 2015). For example, an in vitro murine neural embryonic stem-cell test has been advanced as an alternative for a neurodevelopmental toxicity test (Theunissen et al. 2012; Tonk et al. 2013). The ability to grow rapidly, manipulate, and characterize an array of cell types makes stem cells potentially useful for chemical-toxicity evaluations. Furthermore, assays that use stem cells harvested from genetically diverse populations show considerable promise for providing information that can help in addressing hazard and risk-assessment questions.

Stem cells of potential use in toxicology research are of three primary types: embryonic, adult, and induced pluripotent stem cells. Embryonic stem cells are harvested from embryos that are less than 5 days old and have unlimited differentiation ability. Adult stem cells are isolated from adult bone marrow, skin, cord blood, heart tissue, and brain tissue. Induced pluripotent stem cells (iPSCs) are produced from adult somatic cells that are genetically transformed into a pluripotent state (Takahashi et al. 2007). iPSCs are similar to embryonic stem cells (pseudoembryonic) and can be grown in monolayer and 3-D structures for multiple generations. They can take on a variety of cell types, including neuronal cells (Malik et al. 2014; Sirenko et al. 2014a; Efthymiou et al. 2015; Wheeler et al. 2015), hepatocytes (Gieseck et al. 2014; Sirenko et al. 2014b; Mann 2015), and cardiomyocytes (Sinnecker et al. 2014; Karakikes et al. 2015). The ability to be derived from adult cells and the capacity to differentiate into multiple cell types also make iPSCs particularly promising for exploring human diversity. Cells can be created from specific individuals to produce personalized biomarkers, and iPSCs derived from large patient populations (Hossini et al. 2015; Mattis et al. 2015) could help to identify pathways involved in disease and susceptibility (Astashkina et al. 2012). Because iPSCs are relatively cost-effective to produce on a large scale (Beers et al. 2015), they have the potential to improve cell-based toxicity testing substantially.

There are some challenges to overcome in using stem cells. They can have different expression profiles, which indicate that they might have altered cellular processes, pathways, and functions. Stem cells generally can be difficult to culture and transfect, and the difficulties could limit their application in high-throughput formats. The lack of systematic approaches for characterizing and standardizing culture prac-

tices (such as characterizing cell types, sex origin, and cell function) also presents an obstacle for using stem cells in toxicology applications. Although stem cells (and other cells) have inherent limitations, they are still useful windows into biological processes at the cellular and molecular levels and remain useful for assessing chemical toxicity. A careful evaluation of cell phenotype and properties would help to determine the extent to which human biology is recapitulated in the cellular model.

Modeling Cellular Response

Over the last decade, numerous mathematical models and systems-biology tools have been advanced to describe various aspects of cell function and response. Considerable progress has been made in describing feedback processes that control cell function. The development of cell-based modeling has benefited greatly from coordinated contributions from the fields of cell biology, molecular biology, biomedical engineering, and synthetic biology.

A few simple structural units that have specific functions and appear repeatedly in different species are referred to as network motifs (Milo et al. 2002; Alon 2007). Molecular circuits are built up from network motifs and carry out specific cellular functions, such as controlling cell-cycle progression, xenobiotic metabolism, hormone function, and the activation of stress pathways—the major pathways by which cells attempt to maintain homeostasis in response to chemical and other stressors, such as oxidative stress, DNA damage, hypoxia, and inflammation. Computational models are used to examine those circuits, the consequences of their activation, and their dose–response characteristics.

Toxicity pathways defined in NRC (2007) as cellular-response pathways can be thought of as molecular circuits that, when sufficiently perturbed, lead to adverse effects or toxicity. The circuits can be modeled with computational systems-biology approaches. The tools for describing the circuits and function are developing rapidly (Tyson and Novak 2010; Zhang et al. 2010) and should enable study of the dose–response characteristics of the perturbation of toxicity pathways (Simmons et al. 2009; Zhang et al. 2014, 2015). Quantitative descriptions of the pathways hold the promise of characterizing differences in individual susceptibility to chemicals at the cellular level but will require identification of components of signaling pathways that differ among individuals; sensitivity and other analyses can be applied to determine components that most affect human variability in adverse response. Confidence in the models will increase as they are applied to a more diverse suite of signaling pathways. Model refinement coupled with careful collection of data on detailed biological responses to chemical exposure will test model structures, refine experimental strategies, and help to chart new approaches to understanding of the biological basis of cellular dose–response behaviors at low doses.

TISSUE-LEVEL AND ORGAN-LEVEL RESPONSE

The last decade has seen advances in engineered 3-D models of tissue and computational models for simulating response at the tissue level (see Figure 3-4). This section describes organotypic models, organ-on-a-chip models, and virtual-tissue models that might be particularly applicable for toxicology research.
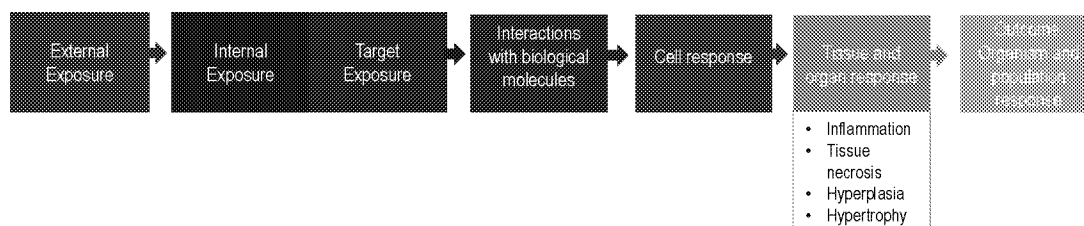

- Inflammation
- Tissue necrosis
- Hyperplasia
- Hypertrophy

FIGURE 3-4 Exposure-to-outcome continuum with example of tissue and organ effects.

Organotypic Models

An organotypic model is a specific type of 3-D culture in which two or more cell types are put together in an arrangement intended to mimic, at least in part, an in vivo tissue and that therefore recapitulates at least some of the physiological responses that the tissue or organ exhibits in vivo. Organotypic models of skin, which contain keratinocytes and fibroblasts, have been developed and validated for use as alternative models for testing skin irritation (Varani et al. 2007), and data from these models are now accepted in Europe for classification and labeling of topically applied products (Zuang et al. 2010). The skin model is being evaluated to improve the specificity of in vitro genotoxicity testing. Organotypic skin cultures appear to have reasonably good concordance with in vivo genotoxicity results (Pfuhler et al. 2014) probably because they retain the ability to metabolize and detoxify chemicals and because the rate of delivery of chemicals to the basal layer is more comparable with the kinetics of dermal absorption in vivo. Other organotypic models include eye, lung epithelium, liver and nervous system tissue (see NASEM 2015). The effects of environmental chemicals have been explored in mouse organoids by using proteomic tools (Williams et al. 2016).

Organ-on-a-Chip Models

An emerging scientific development is the organ-on-a-chip model (see Figure 3-5), which is a 3-D culture grown in a multichannel microfluidic device (Esch et al. 2015). The models are meant to have the same functionality as organotypic cultures but with the ability to manipulate physiological and pharmacokinetic processes (that is, the rate at which a chemical is introduced via the flow-through channels). Several organ-on-a-chip models have been engineered, including ones for liver, heart, lung, intestine, and kidney. The models allow the study of how chemicals can disrupt an integrated biological system and how the disruption might be influenced by the mechanical forces at play in the intact organ, such as the stretching of the alveolar-capillary barrier in lungs due to the act of breathing.
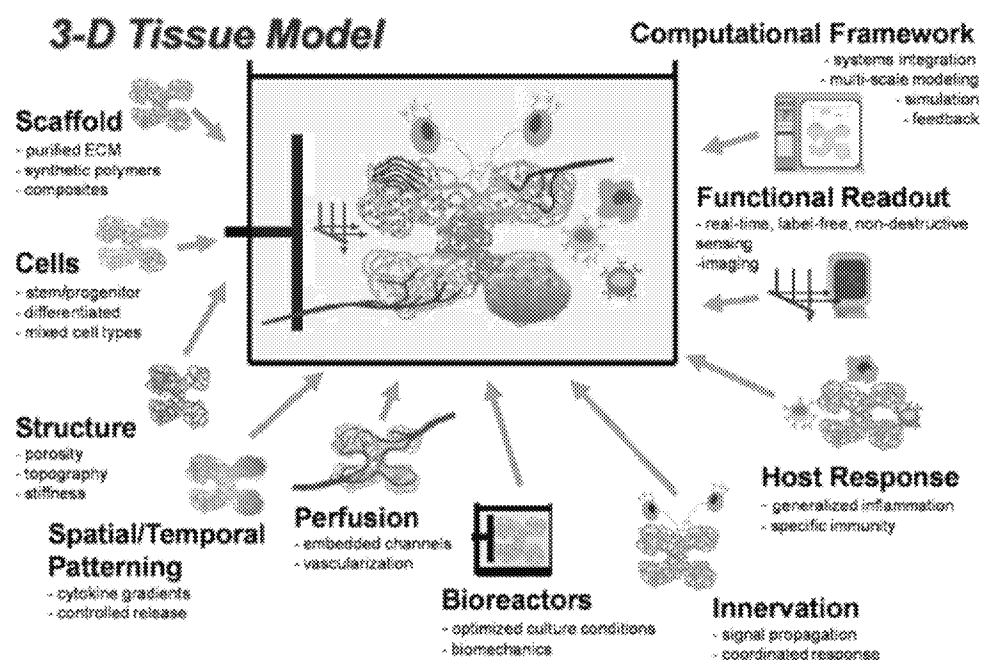

FIGURE 3-5 Generalized components of an organ-on-a-chip model. Source: Birnbaum 2011.

Attempts have been made to design platforms that have different organ mimics arranged in series or parallel that as a system can recapitulate aspects of tissue interactions and in vivo pharmacokinetics (Sung and Shuler 2010). A long-term goal is to introduce a parent chemical into the system and have it move through a liver compartment where it would be metabolized, flow to compartments that contain responsive cell types or to other compartments that contain hydrophobic materials that represent fat, and finally flow through a kidney compartment where it could be eliminated. To date, microfluidic platforms that have that much complexity have not yet been introduced in practice and have not achieved a realistic metabolite distribution through the various tissues in the system (Andersen et al. 2014).

Researchers face challenges in developing such experimental platforms, for example, with the synthetic materials used in the manufacture of the cell-culture substrates. They often are not good mimics of the extracellular matrix and can even absorb small hydrophobic molecules (Wang et al. 2012); that absorption might exert an undue influence on the physiological system or alter chemical concentrations. Large-scale manufacture and high-throughput operation of organ chips also present challenges to the adoption of the technology. Similarly, access to sustainable sources of human cells presents a substantial hurdle for reproducibility and interpretation of the data produced.

Microsystems that are composed of multiple synthetic organ compartments are in the early stages of development, and a number of initiatives are going on to validate model correlations with in vivo observations. For example, the National Center for Advancing Translational Sciences has a number of efforts in this field (NCATS 2016), and the European Union–funded initiative Mechanism Based Integrated Systems for the Prediction of Drug Induced Liver Injury (EU 2015) has also been exploring the use of liver-chip models to predict adverse effects of drugs. Organ-on-a-chip models are promising, but they are not yet ready for inclusion in risk assessments.

Virtual Tissues

As discussed earlier, computational systems biology might be used to describe pathway perturbations that are caused by chemical exposures and the resulting cell responses. Such modeling can be applied to multiple processes that operate in sequence or parallel and used to link cellular responses to tissue-level responses. Modeling feedforward and feedback controls through sequential dose-dependent steps also enable the examination of responses to toxicant exposure that require multiple cell types, such as Kupffer cell–hepatocyte interactions involved in hepatocyte proliferation. Feedback and feedforward control might also contribute to intercellular patterns of response that require input from earlier pathway or cellular functions to activate or inhibit integrated multicellular responses. The cellular responses alter tissue function; the quantitative modeling then focuses on the interface between the cellular-level computation models and virtual-tissue models.

EPA's Computational Toxicology Program has developed mathematical models called virtual tissues for the embryo and the liver (Shah and Wambaugh 2010; Wambaugh and Shah 2010). EPA also has developed a model of blood-vessel development. Virtual-tissue models can use "agent-based" modeling of different cells in the tissue, which relies on and mathematically describes key aspects of cellular behavior or other tissue components to derive the properties of the tissue or organ of interest (Swat and Glazier 2013). The EPA models evaluate chemical exposures that alter growth and phenotypic characteristics of the agents in the models, which in this case are the cells. The models can describe cell growth or pattern formation of different structures in the virtual embryo or regional distribution of cell response in the virtual liver.

As with any model, a critical consideration in developing response models is fidelity of biology between the modeled outcome (virtual-tissue responses) and the apical and other responses observed experimentally. Assumptions and predictions of the models can be tested by using information from human cells and co-cultures with different human cell types. Short-term targeted animal studies that use toxicogenomic tools and other approaches can be used to evaluate the model more broadly. Virtual-tissue models have the potential to help in conceptualizing and integrating current knowledge about the factors that affect key

pathways and the degree to which pathways must be perturbed to activate early and intermediates responses in human tissues and, when more fully developed, to support risk assessments based on studies of key events and how the key events combine to cause adverse responses at the organism level.

ORGANISM-LEVEL AND POPULATION-LEVEL RESPONSE

The Tox21 report (NRC 2007) emphasized a future in which routine toxicity testing would rely on in vitro assays with human cells or assays that probe molecular responses of human toxicity pathways and pathway components. But, the report also noted that in some cases testing in whole animals might be necessary, depending on the nature of the risk-assessment questions, although whole-animal studies were not intended to provide routine information for assessing risks. The need for different types of information related to the nature of the question posed was also emphasized in EPA's report on next-generation risk assessment (EPA 2014b; Krewski et al. 2014; Cote et al. 2016). That report considered three types of assessments: screening and priority-setting assessments, limited-scope assessments, and in-depth assessments. The last one would likely involve a wide array of toxicity-testing approaches, including whole-animal studies. Approaches for assessing variability could also benefit from rodent panels that capture population variability and panels of human cells derived from a group of diverse people. As is true of toxicity-testing tools at the molecular and cellular levels, there has been continuing development of new methods for examining responses in whole animals that are likely to provide important information for the limited-scope and especially for the in-depth assessments. The approaches for assessments on different levels emphasize a fit-for-purpose orientation of designing the testing assays or batteries that depend on the risk-assessment question. This section discusses novel animal models that provide opportunities for enhancing the utility and power of whole-animal testing. It also describes recent advances in structure-based computational models and read-across approaches that provide opportunities for predicting response of data-poor chemicals at the organism level. Figure 3-6 highlights some organism-level and population-level responses.

Novel Whole-Animal Models

Advances in genetics, genomics, and model-organism development have led to genetically well-characterized whole-animal models, including transgenic rodent lines, isogenic mouse strains, and alternative species, such as zebrafish and *Caenorhabditis elegans*, which can be studied in a high-throughput format. Those models coupled with toxicogenomics and novel imaging offer improvements over the traditional in-life rodent studies in that they offer new ways to explore chemical interactions at tissue and cellular levels. Isogenic strains also offer new opportunities to identify determinants of human susceptibility, especially when coupled with new interrogation tools, and to define new mechanisms of toxicity. Targeted testing, which is typically hypothesis-driven and more focused than historical testing strategies, can help to develop and enhance the value of the new animal models, as well as traditional ones. It can be used to explore the mechanisms by which a chemical causes toxicity, how outcomes might differ by age and sex, and how susceptibility might vary in the population. It can help to address specific knowledge gaps in risk assessment and can link in vitro observations to molecular, cellular, or physiological effects in the whole animal. Targeted testing will be critically important in evaluating and validating the robustness and reliability of new computational models, in vitro assays, and testing batteries (Andersen and Krewski 2009; Krewski et al. 2009). As this section shows, the new animal models and outcome-interrogation tools might provide broader assessment of hazards in whole organisms.

Transgenic Rodents

The development of transgenic mouse lines (such as knockin, knockout, conditional knockout, reporter, and humanized lines) advanced biomedical research; a few transgenic rat lines are also available
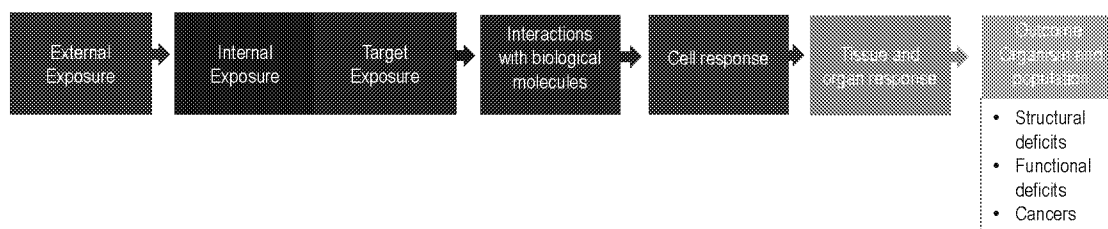
- Structural deficits
- Functional deficits
- Cancers

FIGURE 3-6 Exposure-to-outcome continuum with examples of organism and population-level responses.

now. Novel gene-editing technologies, such as CRISPR/Cas9, have the potential to generate inducible gene editing in adult animals and the creation of transgenic lines in nontraditional mammalian models (Dow et al. 2015). Gene editing permits the creation of experimental approaches that are more specifically suited for various tasks, including targeted testing of susceptible strains and exploration of gene–environment interactions.

Although transgenic animals have been available for decades (Lovik 1997; Boverhof et al. 2011), testing in transgenic animals and incorporation of data from transgenic models into risk assessment has been limited, partly because of questions about applicability for risk assessment and concerns about the cost to develop the models and evaluate a chemical in multiple strains. The National Toxicology Program (NTP) continues to evaluate and develop such models. For example, NTP is using transgenic mice in the testing of the artificial sweetener aspartame, which generally tested negative in standard assays but showed a slight increase in brain tumors in a more sensitive transgenic-mouse strain. The transgenic p16 model was used because it was thought to be susceptible to brain glial-cell tumors. NTP is also testing aspartame in transgenic strains with knocked-out tumor-suppressor genes and activated oncogenes to improve characterization of susceptibility and risk related to gene–environment interactions. Transgenic-rodent mutation data have been used by EPA to understand carcinogenic mechanisms of several agents, such as acrylamide (EPA 2010), but beyond those applications their incorporation into risk assessment has been limited. They have been somewhat more widely used to test specific hypotheses about mechanism, such as the mechanism of liver-cancer induction by phthalates (Guyton et al. 2009), and to evaluate the depth of biological understanding to apply fully organotypic, computational systems-biology, physiologically based pharmacokinetic (PBPK), or other tools.

Genetically Diverse Rodents

Historically, toxicity testing has used only a few rodent species and strains. Although there are advantages in using a well-characterized strain of mice or rats to test chemical toxicity, there are many shortcomings, including concerns about inadequately accounting for profound strain differences in chemical sensitivity and metabolism (Kacew and Festing 1996; Pohjanvirta et al. 1999; De Vooght et al. 2010) and inadequate genetic and phenotypic diversity. High rates of spontaneous disease in some strains (outbred and inbred) can sometimes complicate the interpretation of results. For example, the incidence of background cardiomyopathy in the Sprague Dawley rat can be as high as 100% (Chanut et al. 2013), some strains are completely resistant to some toxicants (Shirai et al. 1990; Pohjanvirta et al. 1999), and it is unclear a priori whether the standard strain has sensitivity that is adequate or too high for identifying a potential human hazard.

Assessment in multiple strains that have known genetic backgrounds is one approach to address variable sensitivity among relatively homogeneous test strains and to address questions related to interindividual sensitivity to toxicants. Initiated in 2005, the Collaborative Cross (CC) is a large panel of novel recombinant mouse strains created from an eight-way cross of founder strains that include three wild-derived strains. The CC has a level of genetic variation akin to that of humans and captures nearly 90% of the known variation in laboratory mice (Churchill et al. 2004). Outbred progeny that have completely reproducible genomes can be produced through the generation of recombinant inbred intercrosses (RIX)

(Zou et al. 2005). Because the CC strains and, by extension, the RIX lines have a population structure that randomizes existing genetic variation, these models provide the increased power that is required to explore the genetic underpinnings of interindividual susceptibility. For example, the CC mouse replicated human susceptibility, immunity, and outcome of West Nile virus infection more comprehensively than the standard inbred model (C57BL/6J) (Graham et al. 2015).

There are several examples of the value of the CC in toxicological evaluation. Trichloroethylene (TCE) metabolism, for example, varies considerably among people and among mouse strains, and the metabolites differ in their mechanisms, toxicity, and organ-specific effects (NRC 2006). That variability has been a critical barrier to understanding of the risk that TCE poses to humans. To address the challenge in TCE-toxicity testing, a battery of mouse lines was used to assess interindividual variability in TCE metabolism and toxicity in the liver and kidney (Bradford et al. 2011; Yoo et al. 2015a,b). Significant differences in toxicity and metabolism were observed in the different strains. Population PBPK modeling was applied to the study results to illustrate how data on diverse mouse strains can provide insight into pharmacokinetic variability in the human population (Chiu et al. 2013).

Multistrain approaches have also revealed fundamental mechanisms of hepatotoxicity of acetaminophen and biomarkers of this potentially fatal effect. Harrill et al. (2009) used a panel of 36 inbred mouse strains and found that liver injury induced by acetaminophen was associated with polymorphisms in four genes, but susceptibility to hepatotoxicity was associated with yet another, CD44. Follow-up study of two healthy human cohorts showed that variation in the human CD44 gene conferred susceptibility to acetaminophen liver toxicity. This powerful example shows how a diverse animal population (in this case, mice) can be used to characterize and identify potential susceptibility in humans.

The Diversity Outbred (DO) population is a heterogeneous stock seeded in 2009 from 144 independent lineages from the CC breeding colony. Each DO mouse is unique and has a high level of allelic heterozygosity (Churchill et al. 2012). Because they were derived from the same eight strains as the CC mice, their genome can be reconstructed with a high degree of precision—a feature that facilitates genome-wide association studies and other similar approaches. A 2015 NTP proof-of-concept study that used DO mice to capture variation in benzene susceptibility successfully identified two sulfotransferases that modify and eliminate benzene metabolites that confer resistance to benzene toxicity (French et al. 2015).

One caveat in using genetically diverse rodent models is that their use potentially can increase animal use. The most effective use of such models in toxicology requires acceptance of novel computational approaches, experimental designs, and statistical approaches that are specifically suited for the models and capable of handling the unprecedented amount of data that these studies generate (Festing 2010). For example, factorial designs can maximize genetic diversity and reduce the risk of false negatives without necessarily requiring more animals than traditional rodent studies to address the central question. Additionally, using DO mice requires accepting that each individual is unique and that there is no way to incorporate "biological replicates" in the traditional sense. Researchers and risk assessors need to be aware of and comfortable with the suite of data that results from these studies and to understand how to integrate the data with information from other sources, including more traditional animal models (see Chapter 7). Computational tools uniquely suited for these emerging animal models are available and readily adaptable to toxicological testing (Zhang et al. 2012b; Morgan and Welsh 2015). Tools for data analysis, visualization, and dissemination are also available (Morgan and Welsh 2015). As with any model system, these rodent models should be used only for questions that they are best suited to address. NTP and other groups are developing frameworks and use cases to highlight when it is advantageous to use such models, and the committee supports further discussion on this issue.

Other Whole-Animal Systems

Advances in genomics, imaging, and instrumentation have made some alternative species—such as *Caenorhabditis elegans* (a nematode), *Drosophila melanogaster* (a fruit fly), and *Danio rerio* (the zebrafish)—useful animal models for hazard identification and pathway discovery. Many technical ad-

vantages are shared among the three dominant nonmammalian species, but zebrafish have several useful characteristics not shared by the others. The genomes of zebrafish and humans display remarkable homology with an overall conservation of over 70%. Furthermore, 80% of the genes known to be involved in human disease are expressed in zebrafish (Howe et al. 2013b). The signal-transduction mechanisms, anatomy, and physiology of zebrafish are homologous to those of humans (Dooley and Zon 2000), and zebrafish have all the classical sensory pathways, which are generally homologous to those of humans (Moorman 2001; Colley et al. 2007).

Another important attribute that might make zebrafish particularly well suited for translational research is the capacity to generate transgenic reporter lines that express fluorescent genes in specific cells, tissues and organs. The large collection of transgenic fish lines are curated by the Zebrafish Model Organism Database and maintained by the Zebrafish International Information Network (Howe et al. 2013a). There is also a rich diversity of zebrafish-disease models and drug screens to help to understand, prevent, and develop therapies for human diseases, including various cancers (Feitsma and Cuppen 2008; Nguyen et al. 2012; Gallardo et al. 2015; Gordon et al. 2015), diabetes and obesity (Gut et al. 2013; Dalgin and Prince 2015; Schlegel and Gut 2015), psychiatric conditions (Panula et al. 2010; Norton 2013; Jones and Norton 2015), heart disease (Arnaout et al. 2007; Chico et al. 2008; Arnaout et al. 2014; Asnani and Peterson 2014; Walcott and Peterson 2014), neurodegenerative syndromes (Bretaud et al. 2004; Chapman et al. 2013; Mahmood et al. 2013; Da Costa et al. 2014; Martin-Jimenez et al. 2015; Preston and Macklin 2015), autism (Tropepe and Sive 2003), immunodeficiencies (Meeker and Trede 2008; Cui et al. 2011), and blood disorders (Ablain and Zon 2013). Zebrafish have been used to investigate neurotoxicants (Levin et al. 2007; Egan et al. 2009; Irons et al. 2010), and Box 3-1 provides an example of using zebrafish for behavioral assessments.

BOX 3-1 Using Zebrafish to Assess Behavior

A limitation of current in vitro screening is the general paucity of assay coverage to identify neurotoxic chemicals reliably. Observations of zebrafish embryonic and larval photomotor responses provide robust measures of nervous-system deficits based on well-established methods. For example, 18–24 hours after fertilization (embryo stage), the photomotor response is measured as tail flexions before and after a bright-light impulse. That assay has proved to be a highly sensitive chemical-toxicity screening tool (Kokel et al. 2010; Reif et al.2016). At 5 days after fertilization (larval stage), the photomotor response can be assessed as a change in swimming activity in response to a sudden light–dark transition. Both tasks can be digitally measured in individual wells, so these complex behavioral assays are highly amenable to high-throughput analysis (Padilla et al. 2012; Truong et al. 2014). The adult zebrafish is increasingly used to measure neurobiological end points affected by chemical exposures. An array of behavioral tests have been designed to probe different domains involved in sensorimotor systems, cognition, and responses related to learning, memory, and anxiety. Indeed, zebrafish adults and juveniles display a variety of complex behaviors, such as kin recognition (Mann et al. 2003; Gerlach et al. 2008), shoaling and schooling (Engeszer et al. 2007; Miller and Gerlai 2012), territoriality (Spence and Smith 2005), associative learning (Al-Imari and Gerlai 2008; Fernandes et al. 2014), and nonassociative responses, such as habituation (Best et al. 2008). A number of neurobehavioral tests of anxiety and exploration have been modeled, and there is some evidence of conserved responses that resemble those of rodent models (Panula et al. 2006; Egan et al. 2009; Champagne et al. 2010; Steenbergen et al. 2011). Startle tests have been developed to understand sensorimotor responses in zebrafish exposed to environmental chemicals. Those assays have been used to test chemical effects on zebrafish motor responses, including responses related to fluorinated organics (Chen et al. 2013), vitamin E deficiencies (Lebold et al. 2013), nanoparticles (Truong et al. 2012), and pesticides (Sledge et al. 2011; Crosby et al. 2015). Collectively, the sophisticated assays could be scaled to increase the throughput with which chemicals are assessed for their effects on the nervous system.

The Zebrafish Mutation Project hosted by the Sanger Institute is yet another major effort that will facilitate cross-species studies. The project aims to develop a knockout allele in every protein-coding gene in the zebrafish genome and characterize its morphological phenotype (Kettleborough et al. 2013). Mining of zebrafish gene or phenotype databases should provide powerful opportunities to identify genes involved in chemical-induced phenotypes.

An additional advantage of zebrafish is that the zebrafish genome is fully annotated, so transcriptomic and all other -omics approaches are possible. Repression of gene expression by antisense morpholinos, siRNA, and such gene-editing techniques as CRISPR/Cas9 is routinely used to assess gene functions in the intact fish, and zebrafish embryos and larvae are nearly transparent, so noninvasive observation is possible. Because larvae measure less than a few millimeters, they can be accommodated in multiwell plates, such as 384-well formats (Rennekamp and Peterson 2015). Only small quantities of test chemicals are needed, so exposure–response relationships can be evaluated over a broad concentration range and testing can be replicated to increase data confidence.

Although substantial research is going on with adult zebrafish for translational research (Phillips and Westerfield 2014; Pickart and Klee 2014), early zebrafish life stages are particularly well suited for rapid screening. During the first 5 days of life, nearly all gene products and signal-transduction pathways are expressed (Pauli et al. 2012); thus, as in other vertebrates, development is a period of heightened sensitivity to chemical exposure. Early–life-stage zebrafish also express a full battery of phase I and phase II metabolism systems, whose activities are highly similar to those of humans (Goldstone et al. 2010).

Despite the advantages of incorporating the use of early–life-stage zebrafish as part of a strategy for making risk-based decisions, there are some noteworthy limitations. First, test chemicals typically are added directly to the aqueous media, not unlike cells in culture. However, the routes of exposure over the course of development, which can affect chemical uptake and metabolism, can be quite different. During the first 2 days of embryonic development, the primary route of exposure is passive dermal adsorption. Later in development, the gills and oral routes become available, and circulation plays a major role in chemical distribution. For the varied routes of exposure, there is little understanding of tissue concentrations, and this contributes to the challenges in comparing concentration–response results in zebrafish with dose–response studies in other systems directly.

A related potential limitation is that despite metabolic similarities to other vertebrates, subtle differences in metabolic activity could lead to inaccurate toxicity predictions, particularly if metabolic activation or inactivation is mechanistically important for specific test chemicals. Because the developing embryo constitutes a comprehensive integrated system, all potential molecular initiating events are operational during testing. Thus, zebrafish are uniquely sensitive to chemical contaminants present in test solutions in that a contaminant could act on biological targets and disrupt critical molecular events. Finally, as with any animal model, the primary sequences of individual pathway components are not necessarily highly conserved. For example, the zebrafish cyclin-dependent kinase 20 (cdc20) protein is 75% identical with the human protein at the amino acid level, and the zebrafish and human aryl hydrocarbon receptors are only 40% identical. In both cases, the homologous proteins are functionally conserved. Although variable conservation of the genomes is a source for potential discordance between zebrafish and humans, the challenge is not unique to zebrafish inasmuch as individual allelic variations between humans can also result in marked differences in chemical susceptibility.

Computational Structure-Based Models for Predicting Organism-Level Response

It has long been recognized that chemicals that have similar chemical structures can elicit the same or similar toxicological effects and that, paradoxically, almost identical chemicals can cause dissimilar biological responses. The extent to which similar chemicals or their metabolites interact with critical biological molecules, such as target proteins, and operate by similar mechanisms is a critical element in determining structure–activity relationships. The last decade has seen advances in the development of structure-based computational methods to predict human health effects. Some are computational expert

systems that consider structural alerts and underlying mechanisms, others are QSAR models that rely on statistical correlations with molecular fragments, and still others are hybrids of these. Many advances have been supported by large curated databases and increased computational power. Health effects addressed include carcinogenicity (Contrera et al. 2005; Valerio et al. 2007), hepatotoxicity (Greene et al. 2010; Hewitt et al. 2013), reproductive and developmental effects (Matthews et al. 2007; Wu et al. 2013), and skin sensitization (Roberts et al. 2007a,b; Alves et al 2015).

The structure-based computational models that are probably the most advanced in model performance and regulatory acceptance are QSAR models for genotoxicity or more specifically for mutagenicity as measured in the Ames assay, a reverse-mutation bacterial assay that is commonly used to evaluate the potential of chemicals to induce point mutations. The development of those models has benefited from the quantity and structural diversity of data available in the public domain on chemicals that have been tested in the Ames assay. As a result of performance, computational models are being accepted as surrogates for actual testing and have recently been incorporated into international guidelines for assessing mutagenic impurities in pharmaceuticals to limit potential carcinogenic risk (ICH 2014). Computational approaches for other human health effects are being considered for use in a regulatory setting (Kruhlak et al. 2012), and the Organisation for Economic Co-operation and Development has published guidance that outlines the needed components of a QSAR model in regulatory settings (OECD 2004). They include "a defined end point; an unambiguous algorithm; a defined domain of applicability; appropriate measures of goodness of fit, robustness, and predictivity; and, if possible, a mechanistic interpretation" (Gavaghan 2007).

The lack of wide use of QSAR models for end points other than mutagenicity might reflect predictive performance that falls short of that required for practical applications. Most approaches predict only whether a chemical will cause the adverse effect. The inability to predict a plasma concentration that would be expected to elicit toxicity ultimately limits utility for differentiating between closely related structures on which little or no safety information is available for comparison.

Read-Across Predictions

Read-across is a process that uses two-dimensional chemical-structure information to identify chemicals (analogues) that have been well studied toxicologically that are then used to predict the toxicity of a similar chemical that has inadequate toxicological data or to group chemicals for the purpose of evaluating their toxicity collectively. Structural similarity can be determined by atom-by-atom matching that results, for example, in a chemical-similarity score or by identifying core molecular structures or functional groups that are thought to be important in conferring toxicity potential. There should also be a consideration of physicochemical similarity among analogues because significant differences in, for example, partition coefficients (such as $logK_{OW}$, a measure of lipophilicity) will have important effects on pharmacokinetic and often pharmacodynamic behavior of a chemical. Read-across approaches are receiving much attention because they can help to satisfy the information requirements under European Union Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations; the general concept has been accepted by the European Chemicals Agency (ECHA) and member-state authorities (Patlewicz et al. 2013). When robust toxicological data are available on one or more structurally related chemicals, they can be used to infer the activity of a chemical that has not been adequately tested. ECHA (2015) has recently published a framework by which it evaluates read-across submissions under REACH. ECHA's framework groups the read-across into six categories according to such factors as whether the read-across is for a single analogue or an entire category, whether it is based on metabolism to a common product, and the relative potencies of members of a chemical series.

Phthalate esters provide a well-studied example of the utility of read-across for male reproductive toxicity. Phthalate esters that have chain lengths of four to six carbons (more if branched) cause testicular toxicity (Foster et al. 1980) and adverse effects on male reproductive-system development (Gray et al.

2000; NRC 2008) in rats. Studies of global gene expression in the fetal rat testis show comparable effects of all the developmentally toxic phthalates (Liu et al. 2005) and support a conclusion that these chemicals act via the same mechanism. Phthalate esters with shorter chains, such as dimethyl and diethyl phthalate, do not produce similar effects on gene expression or on testicular function or male reproductive-system development. Thus, well-studied phthalate esters in this group would serve as anchor chemicals for other phthalates that have chains of four to six carbons in a read-across approach.

Read-across can be problematic, and caution is needed before its conclusions are relied on heavily. For example, thalidomide has two stereoisomers, (S)-thalidomide and (R)-thalidomide, that are virtually identical from a structural perspective in all aspects except for the 3-D orientation of the two ring systems in relation to one another (see Figure 3-7). Their physical characteristics are also identical, so read-across analysis might conclude that the chemicals will have similar or identical safety profiles. However, (S)-thalidomide causes birth defects, embryo death or altered development, growth retardation, and functional defects, whereas (R)-thalidomide does not. Still, the enantiomers are capable of interconverting in vivo, so it is impossible to eliminate the teratogenic effects by administering only the (R)-enantiomer.

Despite the limitations, read-across remains a screening approach for assessing the safety of a molecule in the absence of data on which to base an assessment. The 2015 ECHA framework provides guidance on how protein binding, metabolism, and other data can be used in read-across analyses and potentially overcome the limitations. Furthermore, a recent European study team proposed evaluation of read-across for four basic chemical-group scenarios (Berggren et al. 2015): chemicals that do not undergo metabolism to exert toxicity, that exert their toxicity through the same or structurally similar metabolites, that have low toxicity, or that are structurally similar but have variable toxicity on the basis of their hypothesized mechanism. They have selected chemical groups for case studies in each of the four categories.

Low et al. (2013) extended the concept of similarity in read-across from chemical structure to bioactivity, specifically responses in a variety of in vitro and genomic assays. They proposed a hazard classification and visualization method that draws on both chemical structure and biological features to establish similarity among chemicals in read-across. The approach incorporates mechanistic data to increase the confidence of read-across.

In addition to serving as a screening approach, read-across can be regarded as a hypothesis-generating exercise. The hypotheses can be lumped into two broad categories: the new chemical is metabolized to a chemical that has already been tested (or it and its analogue are metabolized to the same chemical), or the new chemical and its analogues are sufficiently similar in chemical structure and properties that their biological activity is the same (that is, they have the same mechanism). In the former case, there are long-standing methods for assessing chemical metabolism that can be applied to support or refute the hypothesis that the new chemical is metabolized to something that has already been tested. In the latter case, if the mechanism of the analogous chemicals is known, it is reasonably straightforward to test for effects on the initial events of the mechanism (for example, receptor occupancy or enzyme inhibition). In most cases, however, mechanisms are not known; in such cases, it is still possible to compare the responses of the chemical and its analogues in screening systems that globally assess toxicological responses. Global gene-expression analysis is likely to provide universal coverage of possible mechanisms. Gene expression in an animal model in which the target tissues (for the tested analogues) are known or in an in vitro system that represents the target tissue is a reasonable way to test the hypothesis of a comparable mechanism among analogues. It still might be possible to use gene expression in in vitro models to identify a mechanism when target tissue is not known, but it will probably require testing in more than one cell type. Lamb et al. (2006) evaluated the gene-expression changes elicited in four cell types by a large number of drugs; they clearly showed the connections between agents that have the same pharmacological action and demonstrated that this approach has high potential for toxicology. High-throughput screening batteries, such as ToxCast, might also have utility for that purpose, but it will need to be determined
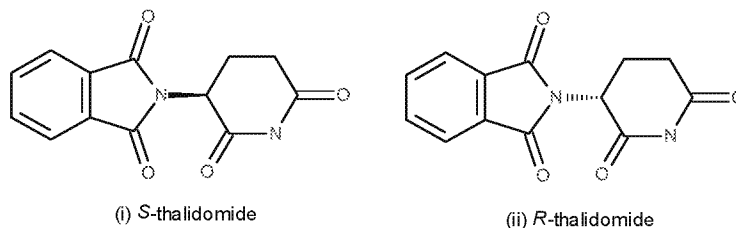
(i) *S*-thalidomide   (ii) *R*-thalidomide

FIGURE 3-7 Molecular structures of (*S*)-thalidomide and (*R*)-thalidomide.

whether the current battery covers the universe of known toxicity mechanisms. Higher-order models, such as organ-on-a-chip or zebrafish, might also be used for testing hypotheses of biological similarity if it can be shown that these models have the biological machinery that is critical for the mechanism in question. As data streams are added more systematically to the read-across process, integrated approaches, such as Bayesian models, that provide for a more agnostic evaluation and promote consistency in output could be developed. Figure 3-8 illustrates several scenarios for read-across and how it can be used to infer hazard and dose–response relationships.

INCORPORATING DATA STREAMS

Various chemicals will have multiple data streams along the exposure-to-outcome continuum that can be used to characterize hazard or risk. For example, pharmacokinetic studies might point to tissues that have particularly high concentrations of a chemical that are potentially increased by active transport as indicated in in vitro studies. Cell-free assays might suggest a set of key receptors, with cell-response assays indicating response; the results, when considered in the context of high concentrations of a chemical in tissues, might indicate particular hazards, such as particular cancers or reproductive toxicity. Targeted studies might show early markers of effect histopathologically, and gene expression in the studies might show consistency with the findings of cell-based assays. The results might be supported by findings on similar chemicals that predict the activity through structure–activity analyses. Robust assessments will identify the more influential data streams with which to develop an integrated assessment. Some streams will be more information-rich than others. The integration of multiple data streams is discussed further in Chapter 7.

CHALLENGES AND RECOMMENDATIONS FOR ADVANCING TOXICOLOGY

This chapter shows how emerging scientific tools generate toxicological evidence on hazard and dose–response relationships of chemicals and other risk issues. It emphasizes how the tools apply to different components in the exposure-to-outcome continuum. Some tools, such as PBPK and systems-biology models, provide a basis for linking components along the continuum. Others, such as high-throughput assays or targeted testing, provide a direct readout of chemical effect within a single component or in multiple components. The tools vary in their maturity for application, their scope of applicability among chemical classes, and the questions that they can address. The committee emphasizes that the level of performance required for the various tools will depend on the question that is being addressed (context) and on agency policies.

There are specific technical and research challenges. Some have been mentioned in preceding sections of this chapter; the challenges related to molecular and cell-based assays are particularly notable. Some important challenges in advancing the tools for risk-assessment application are described below, and some recommendations are offered.

FIGURE 3-8 Scenarios for conducting read-across.

Advancing the New Testing Paradigm

Challenge: Obtaining the vision described in the Tox21 report in which traditional whole-animal testing is replaced with a broad toxicity-testing strategy that uses primarily in vitro assays, computational methods, and targeted animal testing for assessing the biological activity of chemicals is a complex and labor-intensive task that requires focus, commitment, and resources (NRC 2007). The strategy for achieving the vision involves research to understand the spectrum of perturbations that could result in human toxicity and the nature and extent of the toxicity caused by the perturbations and research to understand how determinants of human variability (for example, underlying nutritional, genetic, or disease state or life stage) and exposure duration might affect biological responses or toxicity. The scientific community needs to recognize that the current approach to toxicity testing and data analysis is often compartmentalized, and this prevents a holistic approach in trying to determine toxicity of chemical exposure.

Recommendation: Broad consideration of research that is needed to advance the development of a suite of tests that essentially achieves the vision in the Tox21 report is beyond the present committee's charge, but the committee notes that the research described above in the challenge statement should have high priority so that the vision can be achieved. The committee expresses its concurrence with the Tox21 committee and emphasizes that testing should not be limited to the goal of one-to-one replacement but rather should extend toward development of the most salient and predictive assays for the end point or disease being considered.

Optimizing Tools to Probe Biological Response

Challenge: Developing a comprehensive in vitro system that covers the important biological responses to chemical exposure that contribute to human adverse health effects is a considerable challenge.

Most assays used in the ToxCast program were developed to meet the needs of the pharmaceutical industry and were not designed to cover the full array of biological response, given the extensive testing in whole animals and humans that is conducted for drug development. Thus, not all major forms of toxicity are captured in the current assays, and correlating tested activities with toxicity-hazard traits has been limited. For example, few or no ToxCast or Tox21 assays test for several of the key characteristics of carcinogenesis (Smith et al. 2016). There is also the question of how short-term assay exposures are related to chronic exposure or developmental exposures in vivo. Responses that depend on higher levels of biological complexity could be missed by cell-based assays. A number of issues for assay development acknowledged in NRC (2007) remain, including coverage of the necessary biological space to ensure that human sensitivity and susceptibility to toxicants are adequately captured.

Recommendation: Whole-animal testing should move beyond standard approaches, including those associated with experimental design and statistical methods, to maximize their utility. An array of whole-animal tools are now available, and their adoption could address knowledge gaps in risk assessment more comprehensively and begin to address the breadth of genetic sensitivity in response to chemical exposure and other contributors to human variability in response. Guidance for incorporating these whole-animal tools into risk assessment would likely speed their adoption and use.

Recommendation: Use of targeted rodent tests that incorporate the use of -omics technologies, such as sentinel-tissue transcriptomics, should be encouraged. The experimental design should include strategies for data interpretation and analysis, such as Bayesian approaches, that are specifically developed for these studies. Strategic whole-animal testing could help to identify the broader suite of pathways that are beyond the scope of current molecular and cell-based tests, guide the development of in vitro assays that could enhance confidence in extrapolating from in vitro tests to whole-animal responses, and provide a stronger basis of hazard identification and dose–response assessment.

Recommendation: Tools for probing genomic, epigenetic, transcriptomic, proteomic, and metabolomic changes in cells should be advanced because they provide an opportunity to assess cellular changes in a nontargeted and non–pathway-specific manner. Because virtually all toxicity is accompanied by specific changes in gene expression (and presumably changes in protein expression and metabolic profile), continued exploration of these in vivo and in vitro approaches as standalone screens or as complements to in vitro screens might be a way to cover more biological space.[5]

Understanding and Addressing Limitations of Cell Systems

Challenge: Substantial progress has been made in developing and adapting a wide array of assays for screening environmental chemicals, but cell cultures have several important limitations. There are challenges in incorporating metabolic capacity into the assays to ensure that assay conditions generate chemical exposures that are representative of the exposures in humans that could lead to toxicity. Cell cultures also tend to be extremely sensitive to environmental conditions; changes in microenvironments can alter cellular phenotypes and responses and result in skewed results of toxicity screens. Furthermore, conventional monolayer cultures are less sensitive than 3-D cultures, and the response obtained from an in vitro assay can depend on the cell type that is used—a liver cell vs a neuron or a primary cell vs an immortalized cell. Current in vitro assays evaluate only chemicals that have particular properties; chemicals typically must be soluble in dimethyl sulfoxide, have low volatility, meet molecular-weight cutoffs, and be available in high enough quantity and purity.

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[5]If in vitro methods are used for this purpose, it will be important to identify the minimum number of cell types necessary for full coverage. Identifying the cell types will require a combination of statistical approaches that retrospectively analyze the available transcriptomic data and prospective experimentation to determine the number of cell types that are responsive to a broad array of mechanisms. High-content imaging techniques that capture effects on multiple cellular-toxicity indicators simultaneously—including mitochondrial integrity, cell viability, lipid accumulation, cytoskeletal integrity, and formation of reactive oxygen species (Grimm et al. 2015)—can also be used for nontargeted screening and offer the potential to integrate multiple aspects of cell function.

Recommendation: Formalized approaches should be developed to characterize the metabolic competence of assays, to determine for which assays metabolic competence is not an essential consideration, and to account for the toxicity of metabolites appropriately. Approaches could include the development and application of better in silico methods for predicting metabolism and elimination and the development of methods for including metabolic capability without compromising other aspects of assay performance. Federal agencies have initiated some research to address the metabolic-capacity issue, and the committee recommends that the research have high priority.

Recommendation: Research should be conducted to understand the breadth of cell types needed to capture toxicity that might occur only with specific cell lines. It is possible to identify common pathways of toxicity that exist in all cell types, but biology specific to cell types could be of great use in identifying organ-specific toxicities.

Recommendation: Cell batches—even those from established cell lines—should be characterized sufficiently before, during, and after experimentation. Genetic variability, phenotypic characteristics, and purity should be reported in published literature or on publicly accessible Web sites or interfaces.

Recommendation: Assay development should be coordinated with development of computational models of cellular responses involved in pathway perturbations to promote deeper understanding of shapes of dose–response curves at the cellular level.

Addressing the Whole Human and the Human Population

Challenge: The exposure-to-outcome continuum in reality can be complex. Chemicals can perturb multiple pathways and lead to various forms of toxicity. Furthermore, toxicity can be influenced by genetics, diet, lifestyle choices, social factors, sex, life stage, health status, and past and present exposures. All those factors can influence responses at different points in the exposure-to-outcome continuum and occur in the exposure milieu and context of human experience.

Recommendation: Efforts to capture human variability better in in vitro and in vivo toxicity tests should be explored. Broader testing of multiple cell lines from diverse human populations could find idiosyncratic sensitivity of some populations, as has been seen in in vivo testing of panels of isogenic mouse strains, although this approach addresses only variability due to genetic factors for a single upstream end point. Approaches for better characterization of the variety of possible responses to chemicals in food, drugs, or the environment are needed. Experimental approaches could be coupled with computational approaches for better characterization.

Recommendation: Relatively low-cost, rapid molecular and cellular assays should be used to investigate the toxicity of chemical mixtures. Furthermore, humans are not exposed to single chemicals in isolation but instead are constantly exposed to myriad chemicals in their environment, endogenous chemicals produced in the body or modulated as a consequence of social and behavioral factors, and complex chemical mixtures. Cell-based assays can be used to explore at the molecular and pathway level how the addition of a chemical exposure to existing exogenous and endogenous exposures might contribute to risk.

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Advances in Epidemiology

Epidemiology is the study of health and disease in populations. Standard definitions of epidemiology emphasize a descriptive component that captures patterns of disease by person, place, and time and an etiological component that identifies causes of disease (Gordis 2013). The descriptive element of epidemiology comprises tracking of health and disease indicators and population risk factors (surveillance). The etiological activities—searching for the causes and determinants of disease—involve primarily case–control and cohort studies. The span of epidemiological research also includes intervention studies, both randomized and nonrandomized in the assignment of preventive measures, such as vaccinations, or other interventions.

This chapter addresses the evolving approaches used by epidemiologists to investigate the associations between environmental factors and human disease and the role of epidemiology in the context of the committee's charge regarding 21st century science related to risk-based decision-making. It does not give an overall introduction to the science of epidemiology; such material is readily available in textbooks and elsewhere. It briefly discusses, however, the role of epidemiology in risk assessment, the evolution of epidemiology, data opportunities now available, and types of biases to consider given the use of Tox21 and ES21 tools and methods. The chapter then focuses on the use of -omics technologies in epidemiology and concludes with some challenges and recommendations.

RISK ASSESSMENT AND EPIDEMIOLOGY

The role of epidemiological evidence has long been established within the risk-assessment paradigm originally described in the report *Risk Assessment in the Federal Government: Managing the Process* (NRC 1983) and in various later reports (Samet et al. 1998). Identification of risk factors for disease and inference of causal associations from epidemiological studies provide important information for the hazard-identification component. Evidence on hazard obtained from epidemiological studies is given precedence in evidence-evaluation guidelines, including those of the US Environmental Protection Agency and the International Agency for Research on Cancer (IARC). Convincing epidemiological evidence that indicates a hazard is considered sufficient to establish causation, for example, in the IARC carcinogen classification scheme. However, human data are available on only a relatively small number of agents, particularly in comparison with the large number of environmental agents to which people are potentially exposed. In the absence of natural experiments, observational epidemiological studies are the only scientific approach available and ethically acceptable for studying possible effects of potentially harmful agents directly in human populations.

In addition to providing evidence for hazard identification, epidemiological studies can provide understanding of the exposure–response relationship. For some agents, the effects of exposure have been investigated primarily in particular groups of workers, such as asbestos workers, at exposure magnitudes typically much higher than those of the general population, and exposure–response relationships are extrapolated downward, introducing uncertainty. If the needed exposure data on a general population are available, epidemiological studies can provide key information on risk at exposure concentrations relevant to the population at large. For example, air-pollution exposures of participants in large cohort studies, including the American Cancer Society's Cancer Prevention Study 2 and the multiple studies involved in the European Study of Cohorts for Air Pollution Effects (ESCAPE 2014), have been estimated. Although some exposure misclassification is inherent in the case of most environmental and occupational

exposures, there are numerous examples of successful incorporation of epidemiologically based expo-sure–response relationships into risk assessments: ionizing radiation and cancer, particulate-matter air pollution and mortality, arsenic exposure and cancer, and childhood lead exposure and neuropsychologi-cal development. Methods of addressing or correcting for measurement error have been developed; such corrections generally lead to exposure-response curves with steeper slopes (Hart et al. 2015).

Epidemiological studies can also contribute to understanding the exposure–response relationship by identifying determinants of susceptibility if information on characteristics of study participants (such as their age, sex, and now genomes) is available. Data collected for epidemiological research or for popula-tion surveillance can be useful for describing exposure distributions on the basis of questionnaires, moni-toring, models, and analyses of biological specimens.

Epidemiological research might also provide information on overall population risk that fits into the risk-characterization component of risk assessment. The population attributable risk statistic, originally developed to estimate the burden of lung cancer caused by smoking, provides an estimate of the burden of disease resulting from a causal factor (Levin 1953). Thus, data on human populations can contribute to all four components of the risk-assessment paradigm described in Chapter 1.

EPIDEMIOLOGY IN THE 21st CENTURY

The Evolution of Epidemiology

The methods of epidemiological research have not been static. Initially, epidemiological research on the etiology of noncommunicable diseases—primarily cancer, cardiovascular diseases, pulmonary diseas-es, and metabolic diseases—focused on particular risk factors; exposure assessment was accomplished largely by using self-report questionnaires, measurement and estimation methods in the case of occupa-tional studies, and relatively crude indicators in the case of environmental exposures. Some studies incor-porated measurements from biological samples, such as lead or cadmium concentrations, and some esti-mated exposures with models that used extensive data. For example, in the study of survivors of the Hiroshima and Nagasaki atomic bombings, radiation dose was estimated with an elaborate algorithm that incorporated such information as location and body position at the time of the blast. Epidemiological studies of noncommunicable disease, carried out beginning in the 1950s, focused on risk factors at the individual level; some later studies began to incorporate risk determinants at higher levels of social or organizational structure, including the family, the places of residence and work, and the state and country. Efforts were made to build the studies around conceptual frameworks that reflected understanding of structural, sociological, and cultural factors driving health status and disease risk, and recent decades have seen increasing emphasis on life-course approaches that acknowledge the importance of early life expo-sures, even in utero and transgenerational, for disease risk. Furthermore, many later studies of the envi-ronment and health have been designed to reflect the variation in environmental exposures among and within communities.

Most recently, epidemiological research has been greatly affected by advances in other fields. The start of the 21st century was characterized by rapid advances in technology, medical sciences, biology, and genetics pertinent to epidemiology (Hiatt et al. 2013). Enhanced computing and data-storage capacity have been critical. The advent of genomics and genome-wide association studies (GWASs), for example, has played an important role in promoting the transformation of the practice of epidemiology.

The need to achieve samples large enough to provide studies that have adequate statistical power and the need to replicate novel findings in independent study populations facilitated the evolution of large epidemiological research teams, multicenter studies and consortia, meta-analytical tool development, and data-sharing etiquette. Recent decades have seen an evolution from single investigative teams that have proprietary control of individual datasets and specimens to the establishment of research consortia that have adopted a team-based science and a reproducibility culture through greater sharing of data, proto-cols, and analytical approaches (Guttmacher et al. 2009; Tenopir et al. 2011). Indeed, some funding agen-cies have sought to catalyze the transformation further by supporting the development and dissemination

of validated state-of-the-science protocols designed to ascertain a broad array of phenotypic measures so that individual research teams (when designing new studies) might be positioned better to share and harmonize data among multiple studies (PhenX Toolkit NHGRI).

Case–control and cohort studies—the traditional workhorses of epidemiology—will continue to make strong contributions. Case–control studies, in particular, will continue to contribute to timely in-depth examination of people that have specific rare outcomes, such as rare cancers or reproductive outcomes, including specific birth defects. Cohort studies will continue to play an important role in aiding in the delineation of early antecedents of disease and the identification of preclinical biomarkers and risk factors and contribute to the foundation for translational research and precision medicine. Cohort studies, if started early enough, can be informative on the importance of early life exposures and their influence throughout the life course. The committee anticipates an increasing number of cohort studies that integrate treatment and health-outcome information from multiple sources, including information from health-care delivery systems. Studies that incorporate analysis of samples from companion biobanks will become key resources for connecting mechanisms identified in -omics and other assessments to pathogenesis in humans. Availability of more extensive geographical location information would allow incorporation of new and emerging data streams that document physical and social environments of populations on small scales into existing and new studies.

In summary, the factors reshaping the field of epidemiology in the 21st century include expansion of the interdisciplinary nature of the discipline; the increasing complexity of scientific inquiry that involves multilevel analyses and consideration of disease etiology and progression throughout the life course; emergence of new sources and technologies for data generation, such as new medical and environmental data sources and -omics technologies; advances in exposure characterization; and increasing demands to integrate new knowledge from basic, clinical, and population sciences (Lam et al. 2013). There is also a movement to register past and present datasets so that on particular issues data can be identified and combined. There are already models for data aggregation across studies (for example, National Cancer Institute Cohort Consortium and Agricultural Health cohorts), and researchers recognize the need for harmonizing data collection to facilitate future dataset aggregation (PhenX Toolkit NHGRI; Fortier et al. 2010). They are also considering how to create global biobanks (Harris et al. 2012).

New Data Opportunities

Epidemiology has always been a discipline that uses large quantities of information with the goal of identifying risk factors that can be targeted in individuals or populations ultimately to reduce disease morbidity and mortality. Today, modern technologies—including genomic, proteomic, metabolomic, epigenomic, and transcriptomic platforms and sophisticated sensor and modeling techniques—facilitate the generation and collection of new types of data. The data can be used to generate hypotheses, but they can also be used to supplement data from legacy studies to strengthen their findings (see Box 4-1). New data opportunities have arisen from changes in how medicine is practiced, how health care is delivered, and how systems store and monitor health-care data (AACR 2015). Biobanks are being constructed by a variety of institutions that provide clinical care and potentially constitute new data sources.[1] They typically include collections of biological specimens (blood, urine, and surgical and biopsy specimens), clinical patient information that provides demographic and lifestyle information, perhaps a questionnaire on lifestyle and environmental and occupational exposures, and ascertainment of health outcomes from clinical records. Thus, human data and biosamples potentially available for application of various -omics and

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[1] The committee notes that biobanks are not a new creation. For example, the National Health and Nutrition Examination Survey, which is conducted for surveillance purposes, collects and analyzes specimens, and the data generated have proved invaluable for exposure assessment. Many other population-based biobanks have been created, usually by enrolling healthy subjects; the largest ones include the European Prospective Investigation into Cancer and Nutrition (IARC 2016) and the UK Biobank (2016).

BOX 4-1 Using Legacy Studies

"Legacy" studies have accumulated substantial information on various environmental exposures, such as tobacco use, occupational exposures, and air pollution; personal factors, including genetic data; and disease events that have occurred over decades of follow-up. Some include biological-specimen banks and measures of disease phenotype and intermediate outcomes that were obtained by imaging, physiological testing, and other assessment methods. Some studies have already been used for application of -omics technologies (EXPoSOMICS 2016). Various cohorts have been used to address the association of ambient air pollution with disease incidence and mortality by adding estimates of air pollution at residence locations that were generated by new exposure models that have sufficient spatial resolution. Combining data from multiple studies provides an opportunity to gain statistical power and make results more precise while increasing the variety of exposures and the heterogeneity of study participants.

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other technologies might come from opportunistic studies that rely on data sources that might have been collected and stored for nonresearch purposes. However, evidence from studies that use human tissue and medical data gained through convenience sampling from special populations might not be readily generalized. Furthermore, such studies carry the same potential for bias as other nonexperimental research data, but there is no opportunity with these studies to address some biases via a well-thought out study design, data collection, and protocols for obtaining biospecimens. Thus, new data streams and technologies, although promising, raise important methodological concerns and challenges and are driving the need to develop new study designs and analytical methods to account for technology-specific peculiarities (Khoury et al. 2013). Investigators have cautioned about the increasing possibility of false leads and dead ends with each new assay and have called for careful evaluation of analytical performance, reproducibility, concept validity, and ethical and legal implications (Alsheikh-Ali et al. 2011; Khoury et al. 2013).

The tsunami of data spanning the spectrum of genomic, molecular, clinical, epidemiological, environmental, and digital information is already a reality of 21st century epidemiology (Khoury et al. 2013). There are challenges in using current methods to process, analyze, and interpret the data systematically and efficiently or to find relevant signals in potential oceans of noise. To address those issues, the US government in 2012 announced the "Big Data" Initiative and committed funds to support research in data science in multiple agencies (Mervis 2012). Epidemiologists are poised to play a central role in shaping the directions and investment in building infrastructures for the storage and robust analysis of massive and complex datasets. Given experience with multidisciplinary teams, epidemiologists are also equipped to direct the interpretation of the data in collaboration with experts in clinical and basic health sciences, biomedical informatics, computational biology, mathematics and biostatistics, and exposure sciences. Adaptation of technological advances, such as cloud computing, and strategic formation of new academic–industry partnerships to facilitate the integration of state-of-the-art computing into biomedical research and health care (Pechette 2012) are only some of the initial challenges that must be confronted before new data opportunities can be properly and effectively integrated into future epidemiological studies.

Types of Biases and Challenges Related to External Validity

As noted, contemporary epidemiology is faced with an unprecedented proliferation of clinical and health-care administrative data, -omics data, and social and environmental data. The biases that generally affect epidemiological evidence can be grouped into three broad categories: *information bias* that arises from error in measurements of exposure or outcome variables and co-variates, *selection bias* that arises from the ways in which participants are chosen to take part in epidemiological studies, and *confounding* that arises from the mingled effects of exposures of interest and other exposures. *External validity* refers to the generalizability of findings and is a key consideration in risk assessment. Understanding the selection processes, measurement accuracy, and interpretation of analyses is critical for using epidemiological